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Effect of hydraulic retention time on a continuous biohydrogen production in a packed bed biofilm reactor with recirculation flow of the liquid phase

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Dark fermentation
Anaerobic packed bed reactor
Mesophilic sludge
Hydraulic retention time
Homoacetogenic bacteria

Abstract

The present paper reports on results obtained from experiments carried out in a laboratory-scale anaerobic packed bed biofilm reactor (APBR), with recirculation of the liquid phase, for continuously biohydrogen production via dark fermentation. The reactor was filled with Kaldnes® biofilm carrier and inoculated with an anaerobic mesophilic sludge from a urban wastewater treatment plant (WWTP). The APBR was operated at a temperature of 37 °C, without pH buffering. The effect of theoretical hydraulic retention time (HRT) from 1 to 5 h on hydrogen yield (HY), hydrogen production rate (HPR), substrate conversion and metabolic pathways was investigated. This study indicates the possibility of enhancing hydrogen production by using APBR with recirculation flow. Among respondents values of HRT the highest average values of HY (2.35 mol H₂/mol substrate) and HPR (0.085 L h⁻¹ L⁻¹) have been obtained at HRT equal to 2 h.

Introduction

Currently, sustainable production of fuels is important due to global demand for energy, uncertainty in the supply of petroleum resources and environmental concerns bound up with petrochemicals processing. Biohydrogen, a high energy clean fuel, is considered as a promising alternative to conventional fossil fuels. Hydrogen gas is a recyclable, efficient (energy density equal to 122 kJ/g) and clean fuel with no CO₂ emissions [1–4]. In addition, H₂ can be used as a reactant in hydrogenation processes (in order to produce lower molecular weight compounds), as well as an O₂ scavenger. Due to increasing need for hydrogen energy, in the recent years much progress has been made to determine effective and efficient methods of biohydrogen production.

Many methods to produce biohydrogen have been studied, but most of them are energy intensive and it makes hydrogen production expensive. Currently, about 96% of hydrogen comes from processes based on fossil fuels [5]. Alternative methods of hydrogen generation include electrolysis of water, biophotolysis and biological production. Biological hydrogen production offers the benefits of clean gas, simple technology and is a more attractive potential than the current chemical methods. Hence, generate biohydrogen from renewable source is a promising method, which allows to make
hydrogen a clean and cheap energy carrier. Among the various pathways able to produce hydrogen from biomass, dark fermentation seems to be one of the most attractive processes [6–9]. During dark fermentation biohydrogen and others products are produced via an heterotrophic mechanism in anaerobic conditions, in which carbohydrates are used as the energy and carbon source [10]. It is recognized as an emerging way ahead, because it does not require external energy to drive the process or large surface area to capture the necessary light, it also can use a wide range of substrates, and different pure and mixed cultures [11]. Production of biohydrogen by mixed cultures is preferred from an engineering point of view, because it can be integrated with wastewater treatment systems. Using organic wastes reduces waste disposal problems [12] and it can minimize hydrogen production cost in scaled-up systems [13]. Furthermore, the acids produced during this process (mainly butyric, acetic and propionic acids) can be used for many industrial purposes. Basic dark fermentation provides an economically feasible and environmental friendly process.

Several studies have investigated various sources of carbon, including: sucrose [7, 14–21], glucose [20, 22–27], galactose [28] and fructose [16] as well as different feedstock such as: municipal wastewater [17], yeast factory [3], cheese whey [18, 29, 30] and oat straw hydrolysate [31]. Mix cultures are characterized by better degradation of organic matter and efficiently consume carbon sources compared to pure microbial species [32]. Moreover, hydrogen production using anaerobic organic waste or wastewater can be done without sterilization, which has large economic benefits. Hydrogen yield obtained from mixed culture is generally lower than from pure cultures, due to hydrogen consumption by microorganisms [33]. Thus, inoculum pretreatment is needed and it is one of the most debated issues nowadays. Effective methods of pre-treatment allow to inhibit the methane-producing bacteria activity, sulfidogenic microorganisms, as well as harvest anaerobic spore-forming bacteria. In general, pretreatment methods include: heat [34, 35] and acid shock [35, 36], mechanical pretreatment [37], ultrasonic [38] and electric field [39]. However, the most commonly used method for treatment of mixed culture is heat-shock, which obtains the best performance and higher H2-production rates than acid shock [40, 41]. Furthermore, thermal treatment is simple, inexpensive and effective. It requires temperatures around 100 °C for durations of 15–120 min in order to suppress non-spore-forming bacteria [23, 42–45]. However, the pretreatment at 90 °C for 10 min has also been used [46–48].

In general, hydrogen yield is related to the dominant microorganisms and operating parameters used for fermentation process. It has been demonstrated, that the performance of hydrogen production via dark fermentation is influenced significantly by factors such as pH [23, 49, 50], temperature [50, 51], HRT [3, 14, 17, 18, 23, 24, 27] and hydrogen partial pressure [52]. Specifically, pH has the great influence on hydrogen production, because of it affects on the hydrogenase activity, microbial communities, their structure and metabolism. Therefore, in order to keep medium pH at the optimum value (between 5.5 and 7.8), dark fermentation process has been commonly carried out with pH control systems and buffers such as sodium hydroxide (NaOH), sodium bicarbonate (NaHCO3), hydrochloric acid (HCl) and phosphoric acid (H3PO4) [14–17, 19, 21–27]. However, from an industrial application point of view, hydrogen production without a pH buffer addition offers the major economic and environmental advantages.

Although many efforts have been made to produce hydrogen in dark fermentation, obtained values of hydrogen yield are still low (Table 1). Therefore, improving the efficiency of H2 production poses a major challenge, because it determines the economic viability of the process. Moreover, the improvement in yields of hydrogen production from dark fermentation is a key step towards its commercialization.

Among biological reactors employed in biohydrogen production, anaerobic packed bed reactors (APBRs) are one of the most commonly used. Reactors employing immobilization systems generally show large volumes of biomass accumulation on the support medium [53]. Moreover, maintaining a high biomass inventory in biofilm reactors gives robustness against product inhibition [3]. In comparison to conventional anaerobic treatment systems, biofilm reactors could significantly reduce start-up time and increase organic loading rates up to fivefold [49]. In addition, one of the major advantages of immobilized cell technology is that there is no cell washout at high dilution rates, whereas in continuous stirred-tank reactor, since biomass has the same retention time as the liquid phase, washout of microorganisms can occur at short values of HRT [54]. Also, the construction and operation of packed bed reactors are relatively simple. However, a disadvantage of APBRs is that mixing is not completely achieved, leading to higher mass transfer resistance [55]. Therefore, pH gradient distribution along a reactor column leads to a heterogeneous distribution of microbial activity and thus high hydrogen yield cannot be maintained consistently [56]. To overcome this disadvantage, recirculation flow of a liquid phase can be used. A review of the literature has indicated that studies focused on a long-term hydrogen production via dark fermentation in APBRs, equipped with the system for back-mixing, are limited to only few papers [19, 22, 27]. Fontes Lima and Zaiat [19] have demonstrated the positive effect of a liquid recirculation on H2 production via dark fermentation in APBRs. The aforementioned authors have found that the optimum value of the recycle ratio is equal to 0.5–0.6. Based on this, in Ref. [22] a packed bed biofilm reactor with a liquid recycle (at 60% of the feed flow rate) was applied. In turn, dos Reis and Silva [27] have investigated the impact of HRT (in the range from 1 to 8 h) on hydrogen and ethanol coproduction in anaerobic packed bed reactors equipped with effluent recycling. However, in mentioned papers [19, 22, 27], in order to improve biohydrogen production, pH control systems have been used.

In response to the state of the existing literature, the aim of this study was to evaluate the effect of theoretical hydraulic retention time on hydrogen yield (HY), hydrogen productive rate (HPR) and composition of soluble microbial products in an anaerobic packed bed biofilm reactor equipped with recirculation flow of soluble products, inoculated with a mesophilic sludge, without pH buffer addition.
Table 1 – Hydrogen yield and soluble microbial products obtained in APBRs.

<table>
<thead>
<tr>
<th>Carrier material</th>
<th>Inoculum/pretreatment</th>
<th>Substrate</th>
<th>COD (g/L)</th>
<th>HRT (h)</th>
<th>T (°C)</th>
<th>pH (inlet)</th>
<th>pH control system</th>
<th>HY average (mol H₂/mol substrate)</th>
<th>Recirculation flow</th>
<th>Homoacetogenic activity</th>
<th>Soluble products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-density polyethylene</td>
<td>Natural fermentation/NI</td>
<td>Sucrose</td>
<td>NI</td>
<td>10.2</td>
<td>55.0</td>
<td>6.5</td>
<td>-</td>
<td>1.6</td>
<td>-</td>
<td>NI</td>
<td>HAc, HBu, HPr, HVa, HCP, ETOH</td>
<td>[7]</td>
</tr>
<tr>
<td>Expanded clay</td>
<td>Municipal sewage sludge/Acidic</td>
<td>Sucrose</td>
<td>20.0</td>
<td>0.5–5.0</td>
<td>35.0</td>
<td>6.7</td>
<td>+</td>
<td>0.1–1.1</td>
<td>-</td>
<td>-</td>
<td>HAc, HPr, HBu, ETOH</td>
<td>[14]</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>Municipal sewage sludge/Acidic</td>
<td>Sucrose</td>
<td>20.0</td>
<td>0.5–2.0</td>
<td>35.0</td>
<td>6.7</td>
<td>+</td>
<td>NI</td>
<td>-</td>
<td>-</td>
<td>HAc, HPr, HBu, ETOH</td>
<td>[14]</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>Municipal sewage sludge/Acidic</td>
<td>Sucrose</td>
<td>20.0</td>
<td>1.0–3.0</td>
<td>35.0</td>
<td>6.7</td>
<td>+</td>
<td>NI</td>
<td>-</td>
<td>-</td>
<td>HAc, HPr, HBu, ETOH</td>
<td>[14]</td>
</tr>
<tr>
<td>Plastic rings</td>
<td>Mixed cultures/NI</td>
<td>Sucrose</td>
<td>10.0</td>
<td>2.0–30.0</td>
<td>26.0</td>
<td>7.8</td>
<td>+</td>
<td>0.8–1.2</td>
<td>-</td>
<td>-</td>
<td>HAc, HPr, HBu</td>
<td>[15]</td>
</tr>
<tr>
<td>Polyethylene–octene elastomer</td>
<td>Municipal sewage sludge/Acidic</td>
<td>Sucrose</td>
<td>20.0</td>
<td>4.0</td>
<td>35.0</td>
<td>6.0</td>
<td>+</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>HAc, HPr, ETOH</td>
<td>[16]</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>Municipal sewage sludge/Acidic</td>
<td>Sucrose</td>
<td>20.0</td>
<td>0.5–4.0</td>
<td>35.0</td>
<td>6.7</td>
<td>+</td>
<td>1.2–3.9</td>
<td>-</td>
<td>-</td>
<td>HAc, HBu, HPr, ETOH</td>
<td>[17]</td>
</tr>
<tr>
<td>Ceramic</td>
<td>Soft drink wastewater/NI</td>
<td>Sucrose</td>
<td>10.0</td>
<td>1.5–24.0</td>
<td>55.0</td>
<td>4.5–5.5</td>
<td>–</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>HAc, HBu, ETOH</td>
<td>[18]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Natural fermentation/NI</td>
<td>Sucrose</td>
<td>NI</td>
<td>2.0</td>
<td>25.0</td>
<td>6.5</td>
<td>+</td>
<td>0.9–1.4</td>
<td>+</td>
<td>–</td>
<td>HAc, HBu, HPr, ETOH</td>
<td>[19]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Natural fermentation/NI</td>
<td>Sucrose</td>
<td>2.0</td>
<td>2.0</td>
<td>25.0</td>
<td>NI</td>
<td>NI</td>
<td>0.6</td>
<td>–</td>
<td>+</td>
<td>HAc, HBu, HPr, ETOH</td>
<td>[20]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Natural fermentation/NI</td>
<td>Glucose</td>
<td>2.0</td>
<td>2.0</td>
<td>25.0</td>
<td>NI</td>
<td>NI</td>
<td>1.2</td>
<td>–</td>
<td>–</td>
<td>HAc, HBu, HPr, ETOH</td>
<td>[20]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Anaerobic sludge from UASB reactors/Heat, acidic</td>
<td>Sucrose</td>
<td>2.0–24.0</td>
<td>2.0</td>
<td>25.0</td>
<td>6.5</td>
<td>+</td>
<td>0.7–2.1</td>
<td>–</td>
<td>+</td>
<td>HAc, HBu, HPr, ETOH</td>
<td>[21]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Municipal sewage sludge/Heat</td>
<td>Glucose</td>
<td>2.0–64.0</td>
<td>8.0</td>
<td>37.0</td>
<td>6.5</td>
<td>+</td>
<td>1.0</td>
<td>+</td>
<td>–</td>
<td>HAc, HBu, HPr</td>
<td>[22]</td>
</tr>
<tr>
<td>Low-density polyethylene</td>
<td>Municipal sewage sludge/Heat</td>
<td>Glucose</td>
<td>2.0–64.0</td>
<td>8.0</td>
<td>37.0</td>
<td>6.5</td>
<td>+</td>
<td>2.0</td>
<td>+</td>
<td>–</td>
<td>HAc, HBu, HPr</td>
<td>[22]</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>Municipal sewage sludge/Heat</td>
<td>Glucose</td>
<td>NI</td>
<td>12.0–24.0</td>
<td>37.0</td>
<td>5.0–6.5</td>
<td>+</td>
<td>0.7c</td>
<td>–</td>
<td>+</td>
<td>HLC, HAc, HPr, HBu, HFr, HS</td>
<td>[23]</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>Municipal sewage sludge/Heat</td>
<td>Glucose</td>
<td>8.0</td>
<td>2.0–24.0</td>
<td>37.0</td>
<td>5.7</td>
<td>+</td>
<td>NI</td>
<td>–</td>
<td>+</td>
<td>HLC, HAc, HPr, HBu, ETOH</td>
<td>[24]</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>Municipal sewage sludge/Heat</td>
<td>Glucose</td>
<td>20.0</td>
<td>4.0</td>
<td>40.0</td>
<td>6.0–7.0</td>
<td>+</td>
<td>0.9</td>
<td>–</td>
<td>–</td>
<td>HAc, HPr, HBu, ETOH</td>
<td>[25]</td>
</tr>
<tr>
<td>Inert stone chips</td>
<td>Municipal sewage sludge/Heat, acidic</td>
<td>Glucose</td>
<td>5.0</td>
<td>24.0</td>
<td>28.0</td>
<td>6.0–7.0</td>
<td>+</td>
<td>0.014–0.016b</td>
<td>–</td>
<td>–</td>
<td>HAc, HPr, HBu</td>
<td>[26]</td>
</tr>
<tr>
<td>Expanded clay</td>
<td>Swine slaughterhouse sludge/Heat</td>
<td>Glucose</td>
<td>3.5</td>
<td>1.0–8.0</td>
<td>25.0</td>
<td>4.0–5.0</td>
<td>+</td>
<td>1.2–2.4</td>
<td>+</td>
<td>–</td>
<td>HAc, HBu, HPr, ETOH, MetOH</td>
<td>[27]</td>
</tr>
</tbody>
</table>


a Based on article data.

b mol H₂/g COD consumed.

c Maximum value.
**Materials and methods**

**Reactor design and support material**

Anaerobic packed bed reactor (APBR) with a cylindrical jacketed glass was used for the experiments (Fig. 1). The inner diameter was 10 cm, the height 40 cm and the total working volume 2.1 L. The reactor was filled with Kaldnes® biofilm carrier (10 mm), made by high density PE. The material had a density of approximately 0.95 g/cm³ and a porosity of 90%.

**Heat-treatment of H₂-producing sludge and medium**

The inoculum used in this study was an anaerobic mesophilic sludge obtained from an urban wastewater treatment plant (WWTP) in Aix-en-Provence, France. The bacterial community structures of sludge sampled from this station has already been studied and presented in Ref. [57]. According to this work, we supposed that the sludge used in the present study was the most predominant by phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. The support of this hypothesis is the fact, that members of these phyla have already been found as dominated in mixed anaerobic consortia producing biohydrogen [58–60].

Before seeded into the reactor, the sludge (10% v/v) was heat-treated in anaerobic conditions at 100 °C for 1 h to inhibit the methane-producing bacteria activity and harvest anaerobic spore-forming bacteria [42,43]. It has been demonstrated that *Clostridium* species in heat-treated sludge are the most commonly identified bacteria responsible for biohydrogen production [61–63]. In order to investigate the influence of substrate type on instability in the biohydrogen production, as a carbon source glucose and sucrose have been used (initial concentration: 5000 mg/L) (Table 2). The medium used for biohydrogen production consisted also of 9 following inorganic supplements (mg/L): NH₄Cl, 500; K₂HPO₄, 250; KH₂PO₄, 250; MgCl₂, 300; CoCl₂, 25; CuCl₂, 10; MnCl₂, 15; CaCl₂, 5; FeCl₃, 25. The reactor was operated without any additional reagents for pH adjustment.

**Cell immobilization**

Prior to cell immobilization, the reactor was purged with nitrogen gas for 20 min to ensure anaerobic conditions. 230 mL of pretreated seed sludge with 2.070 L of synthetic wastewater were injected at the inlet of the immobilized reactor. In order to promote adhesion and growth of the biofilm on the carrier surface, the reactor was operated in a batch mode by recirculation of the feeding solution by a peristaltic pump at a HRT equal to 2 h during the first 29.5 h. Circulation used for the purpose of cell attachment was terminated when 70% of biomass was attached to the biofilm carrier. After the activation period reactor was switched on to a continuous mode with a designated theoretical hydraulic retention times, began with 5 h.

**Reactor setup and operating conditions**

The APBR was fed with a synthetic wastewater containing carbon source (glucose/sucrose) and 10% (v/v) of heat-treated sludge. Production of biohydrogen by the immobilized culture was continuously operated. Fresh inlet was fed to the reactor by a peristaltic pump according to the predetermined HRT values. Liquid effluent was collected from the side of the reactor, while the gaseous effluent was collected from the top. Flow rate of the biogas was measured by a glass soap bubble flow meter. In order to decrease dissolved gas (H₂ and CO₂) concentrations and minimize process inhibition as well as remove dissolved oxygen, the reactor was purged with nitrogen every day (100 mL/min, 20 min). This also allowed to the creation of anaerobic conditions. The temperature of the reactor was set at 37 °C by recirculation heated water from a thermostatic bath through the column water jackets.

![Fig. 1 – Packed bed biofilm reactor for continuous biohydrogen production.](image-url)
study was divided into five experimental phases (Table 2) with 14–21 days long each one, corresponding to the theoretical values of HRT from 5 to 1 h. Flow rates in an inlet were equal to: 0.40; 0.66; 1.0 and 2.0 L/h. In order to increase liquid-gas mass transfer, recycle of a liquid phase was applied. Effluent was recycled through a recycle pump connecting effluent outlet and feed inlet. Based on finding presented in Ref. [19] a ratio between flow in the recirculation line and the inlet equal to 0.5 has been applied. Therefore, the recycle flow rates were equal to 0.20; 0.33; 0.50 and 1.0 L/h, respectively, for each theoretical values of HRT. The reactor was operated during 77 days, without addition of an alkalinity agent. The concentration of gas products and soluble metabolites (volatile fatty acids and ethanol) were evaluated during all operation phases at designated time intervals. pH and concentrations of carbon source in the influent and effluent of the reactor were also recorded. The results reported in the present paper are the average values for each phases.

**Analytical methods**

The carbohydrates concentrations of the reactor’s influent and effluent were measured using Standards Methods (via phenol-sulfuric acid method). Concentrations of volatile fatty acids (VFA) and alcohols were also measured by gas chromatography (Agilent Technologies) 7890B GC system equipped with DB-WAX column (30 m × 0.25 mm × 0.25 μm). Before analysis, effluent samples were filtered through a 0.2 μm membrane. The temperatures of the injector and detector were 250 °C and 300 °C, respectively. The oven temperature increased from 100 °C by a ramp-up of 10 °C/min for 5 min, and was held at a final temperature of 250 °C for 12 min. Helium was used as the carrier gas with a flow rate of 3 mL/min. The composition of the gas in the headspace of the reactor was determined by a Varian 3800 Gas Chromatograph. The analyses of solids (total suspended solids – TSS and total volatile solids – TVS) and pH were performed according to Standard Methods (APHA, 1998). A total organic carbon (TOC) analyzer (TOC-V Shimadzu) was used to measure the organic content in the feed solution and the effluent of the reactor. The procedure followed for biofilm quantification was adapted from Standard Methods [64].

**Temperature control**

Since temperature is one of the most important factors which has the significant influence on the activities of hydrogen-producing bacteria and the fermentative hydrogen production, the temperature profile inside the reactor was carefully investigated. Nine T-type thermocouples were used, which were arranged along the vertical axis (H) at 135 mm from each other and along a horizontal axis (r) at 25 mm from each other. Installed positions of thermocouples are shown in Fig. 2. In addition, two thermocouples have been used to measure temperature in the inlet and outlet of the jacket and one in the inlet of the reactor. Temperature was measured with 5-s intervals during 77 days with the accuracy equal to 0.01 °C.

![Fig. 2 – Installed positions of thermocouples for measuring temperature in a packed bed.](image)

**Table 2 – Operational periods during the fermentation process.**

<table>
<thead>
<tr>
<th>Operational phase</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (days)</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Substrate</td>
<td>Glucose</td>
<td>Sucrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT (h)</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Inlet flow rate (L/h)</td>
<td>0.40</td>
<td>0.66</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Recirculation flow rate (L/h)</td>
<td>0.20</td>
<td>0.33</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Flow rate recirculation/inlet (−)</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Results and discussion**

**pH and temperature profile**

pH in the influent and effluent of the reactor was stable (Fig. 3) and equal to 4.49 ± 0.46 and 3.63 ± 0.51, respectively.

The values of average temperature inside the reactor at different axial and horizontal positions are presented in Fig. 4. In general, during 77 days of the fermentation process, the average temperature inside the APBR was equal to 36.60 °C. However, it should be noted, that the specific average value depends on the place inside the reactor and it increases along a height and decreases along a radius of the packed bed. The highest average temperature (37.46 ± 0.86 °C) was noted at the position r = 0 and H = 0.135 m. In turn, the lowest average temperature (35.14 ± 1.28 °C) was observed near to the inlet of reactor (r = 0.025 m and H = 0). The highest difference of average temperature along the horizontal and the vertical axis was equal to 1.74 °C and 2.03 °C respectively. The average values of temperature in the inlet and outlet of the reactor...
were constant and equal to 37.57 °C and 37.55 °C, respectively. The average temperature of the feeding solution after mixing was equal to 25.50 °C.

Biohydrogen production

Instability in the hydrogen production

The produced biogas was composed of H2 and CO2. No methane was detected throughout all the periods of the APBR operation, indicating that the method used for pretreatment of the inoculum leads to effective removing methanogen bacteria. However, hydrogen production fluctuated strongly for both carbon sources (glucose and sucrose) over the range of experimental conditions. Fig. 5 shows obtained values of H2 content in the biogas, HY (mol H2 produced/mol substrate consumed) and HPR (volume H2 produced/H2 evolution time/reactor volume) during all reactor operation.

The content of H2 in the biogas was constant (64.78 ± 2.98%) during the first 9 days of operation and then it drastically decreased to the value 22.94% (21th day of process). Further decreasing values of HRT to 3; 2 and 1 h led to increasing H2 content to values equal to about 45.28%, 71.49% and 60.60%, respectively, but just for short periods of time (3-4 days). In general, systematic decreasing of H2 concentration has been observed throughout the process.

Instability of hydrogen production in APBRs operation has been widely reported in the literature[7,19–21,23,24] and it is a typical issue, which occurs during long-term operations of systems inoculated by mixed bacteria cultures. Penteado et al. [21] have credited the instability to the consumption of H2 and CO2 by methanogenic or sulfidogenic organisms. Because in this study methane and hydrogen sulfide were not detected in the gas phase, the consumption of hydrogen by these organisms should be excluded. The most coherent hypothesis is that this instabilities are related to homoacetogenic microorganisms, which use the Wood-Ljungdahl (or acetyl-CoA) pathway, where CO2 and H2 are converting into acetic acid and water through the following reaction [65].

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (1)$$

This pathway was the main responsible for the hydrogen production instabilities in long term operation of APBRs in studies [19–21,23,24].

Homoacetogenic bacteria are strictly anaerobic and belong to: *Acetobacterium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus* and *Sporomusa*, whereas *Clostridium* and
Acetobacterium sp. are the most common [66]. Although their role in dark fermentation is important, it is still not clear [66]. Moreover, the threshold concentrations of H2 and CO2 required are not well characterized. Oh et al. [67] demonstrated, that heat-shock is able to remove methanogenic strain, but may not remove some strains of homoacetogenic bacteria such as same genus Clostridium, which can survive heat shock due to the ability to form high-temperature resistant endospores. According to [66], no effective method is known, which leads to eliminate hydrogen losses via acetogenesis. It is due to the fact, that homoacetogenic bacteria activity does not depend on their source, pretreatment conditions, substrate, type of reactor as well as process parameters. However, Duangmanee et al. [68] demonstrated, that to maintain stability in continuously hydrogen production repeat pre-treatment of inoculum every day is needed. In turn, maintain stability in continuously hydrogen production is controversial. The optimum values of HRT for these reactors have been reported as 2 and 1 h, respectively. In turn, in studies [15,18] it has been demonstrated, that during H2 production in APBRs, decreasing values of HRT (increasing of the substrate loading rate) leads to increasing H2 content in a biogas, HY and HPR. According to [15,18] the lower H2 content in the biogas at higher values of HRT (3–5 h) was caused by excessive production of CO2 by bacteria species, which do not produce biohydrogen. Moreover, short HRT led to higher substrate flow and thus to increasing the rate of substrate conversion.

The impact of HRT on hydrogen production in APBRs has been extensively investigated and presented in the literature [3,14,17,18,23,24,27]. For example Chang et al. [14] have demonstrated, that HRT strongly affects hydrogen production in two fixed-bed reactors packed with expanded clay or activated carbon. The optimum values of HRT for these reactors have been reported as 2 and 1 h, respectively. In turn, in studies [15,18] it has been demonstrated, that during H2 production in APBRs, decreasing values of HRT (increasing of the substrate loading rate) leads to increasing H2 content in a biogas, HY and HPR. According to [15,18] the lower H2 content in the biogas at higher values of HRT (3–5 h) was caused by excessive production of CO2 by bacteria species, which do not produce biohydrogen. Moreover, short HRT led to higher substrate flow and thus to increasing the rate of substrate conversion.

Review of the literature has indicated that maximum average value of hydrogen yield obtained in this study (2.35 mol H2/mol sucrose) was much higher than average values achieved in APBRs for the same carbon source (sucrose) and reported in several previous papers [7,14–16,19–21]. Moreover, in all of the mentioned studies values of medium pH were kept in the range between 6.0 and 7.8, which is known as favorable for H2 production. For example, Chang et al. [14] for an APBR filled with expanded clay as a support material, operated under HRT between 0.5 and 5 h and pH equal to 6.7, have achieved the maximum average value of hydrogen yield equal to 1.1 mol H2/mol sucrose. Li et al. [15], by applying the wide range of HRT values (from 2 to 30 h) and pH medium 7.8, have obtained the maximum average value of HY 1.22 ± 0.13 mol H2/mol sucrose. In another study, HY of 0.9 mol H2/mol sucrose was achieved in a packed bed biofilm reactor operated under HRT 4 h and pH 6.0 [16]. In turn, Penteado et al. [21] for an APBR operated under HRT 2 h and medium pH 6.5, have noted the maximum value of hydrogen yield equal to 2.1 mol H2/mol sucrose. It has to be pointed out that in the current

<table>
<thead>
<tr>
<th>Substrate</th>
<th>H2 (mol H2/mol substrate)</th>
<th>HPR (L h⁻¹ L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.52 ± 0.23</td>
<td>0.042 ± 0.018</td>
</tr>
<tr>
<td>3</td>
<td>1.65 ± 0.59</td>
<td>0.055 ± 0.047</td>
</tr>
<tr>
<td>2</td>
<td>2.35 ± 1.37</td>
<td>0.085 ± 0.027</td>
</tr>
<tr>
<td>1</td>
<td>1.80 ± 0.83</td>
<td>0.056 ± 0.020</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>68.33</td>
<td>0.067</td>
</tr>
<tr>
<td>3</td>
<td>46.82</td>
<td>0.103</td>
</tr>
<tr>
<td>2</td>
<td>71.49</td>
<td>0.119</td>
</tr>
<tr>
<td>1</td>
<td>60.60</td>
<td>0.121</td>
</tr>
</tbody>
</table>

**Table 3 – Influence of HRT on H2 content in a biogas, HY and HPR during the fermentation process. Mean values ± standard deviation.**
study average value of HY was also higher than that obtained in a thermophilic hydrogen-producing system demonstrated in Ref. [7]. Higher value of HY achieved in our work probably results from the use of recirculation line of liquid products. According to Ref. [19] it led to obtain higher mass-transfer fluxes and thus improvement hydrogen production. Therefore, the findings obtained in this study clearly indicate the possibility of biohydrogen production without using pH buffers in APBRs equipped with a liquid recirculation. Since this solution allows to avoid the use of chemical reagents, it can have the positive impact on environmental and economic aspects of biohydrogen production.

**Composition of soluble products**

The concentrations of soluble metabolites were measured every day during the course of hydrogen production. A predominance of acetic acid (HAc), butyric acid (HBu), propionic acid (HPr) and ethanol (EtOH) have been obtained in all experimental phases (Fig. 6). The same composition of a liquid phase during dark fermentation in APBRs has been observed in several studies [14-16,19,21,25].

During operation, the production of soluble products in the reactor changed. Table 4 shows the average values of the main intermediate products concentration and substrate conversion under different applied values of HRT. It has been observed, that HRT has the significant influence on the average concentration values of acetic and butyric acids, and ethanol. When HRT decreased from 5 to 1 h, production of ethanol and acids: butyric and acetic decreased drastically: from 930 ± 200 to 80 ± 20 mg/L, 720 ± 500 to 80 ± 10 mg/L, 690 ± 110 to 110 ± 10 g/L, respectively. In turn, decreasing of HRT from 5 to 3 h did not affect significantly on the propionic acid concentration (decreasing of the average concentration from 340 ± 30 to 310 ± 10 mg/L). However, further decreasing from 3 to 1 h led to decreasing of propionic acid concentration to 70 ± 10 mg/L. It has been demonstrated, that HRT has also the significant impact on substrate conversion. Decreasing HRT from 5 to 1 h led to decreasing substrate conversion from 70.69% ± 10.27 to 9.59% ± 0.82. This indicates, that low values of HRT reduce the substrate used efficiency. It is due to the fact, that at low values of HRT the substrate residence time in APBR was too short for organic matter degradation. Therefore, for complete substrate fermentation its smaller concentration or higher HRT are required.

There are several pathways for the production of hydrogen, organic acids and EtOH. In general, the determination of the composition of soluble microbial products (SMP) implied the fermentation pathway [47].

In order to evaluate the effect of HRT on the soluble products production, the ratio of each metabolite over the SMP formed was calculated (Table 5). Ethanol was the most common metabolite for HRT between 5 and 2 h (from 34.47% to 45.46% of the SMP), with its concentration ranging between 180 ± 20 and 930 ± 200 mg/L. In general, ethanol is considered as an unfavorable metabolite for biohydrogen production. However, Zhu et al. [69] justified the study with high H2 production with ethanol as a by-product, by suggesting the following pathway

\[
C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2H_2 + 2CO_2
\] (2)

For HRT equal to 1 h dominant was acetic acid (33.77% of the SMP) (Table 5). It is understandable that the productivity of all metabolites tends to decrease with decreasing HRT due to
decreasing substrate conversion (from 70.69% ± 10.27 to 9.59% ± 0.82).

According to [1,2] the HAc/HBu ratio has been used as an indicator of hydrogen production. The acetic pathway is considered as the most effective pathway in dark fermentation process. In general, a higher HAc/HBu ratio gives a higher theoretical H2 yield, according to the following stoichiometric equations

\[
\begin{align*}
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2 & \rightarrow 2\text{CH}_3 + 4\text{H}_2 + 2\text{CO}_2 \\
\text{C}_6\text{H}_{12}\text{O}_6 & \rightarrow \text{CH}_3(\text{CH}_2)\text{COOH} + 2\text{H}_2 + 2\text{CO}_2
\end{align*}
\]

In this study dark fermentation was predominated by the acetic acid pathway. The HAc/HBu ratio increased from 1.42 to 2.50 when the HRT was reduced from 5 to 2 h (Table 4). The results obtained in the present work confirm that the highest HAc/HBu ratio corresponds to the highest biohydrogen yield (Fig. 7). The highest values have been obtained at HRT equal to 2 h (Fig. 7).

**Cell washout and biofilm analyses**

In order to investigate a successful immobilization, total suspended solids (TSS) in the effluent of the APBR were periodically measured. During all analyzed operational conditions small biomass amount in the effluent of the reactor has been found. It indicates the robustness of the APBR against cell washout in the continuous biohydrogen production. Moreover, it has been demonstrated, that HRT has the impact on TSS amount in the reactor effluent. In general, decreasing value of HRT led to increasing the amount of biomass leaving the reactor (Fig. 8). This phenomenon is due to the fact, that decreasing HRT from 5 h to 1 h (HRT = 4 h was not applied in the present study) was related to increasing the flow velocity in the reactor inlet from 0.40 L/h to 5 L/h. Thus, much more intensive washing out of bacterial cells has been observed.
In order to analyze biofilm quantification, after the experiments, the followings steps were performed:

- **Sampling**: a known mass of plastic beads samples with attached biofilm was taken respectively from the bottom, half-height and the top of the reactor.
- **Separation of the biofilm from plastic beads**: the plastic beads were introduced in a vial with distilled water and sonication was performed to separate the biofilm from plastic beads.
- **Calculation of TSS and TVS**: the extracted biofilm sample was dried at temperature equal to 105 °C for 24 h (TSS) and then at 550 °C for 2 h. The amount of biofilm attached to carrier material was calculated as a difference of weight between samples dried at those two temperatures and expressed as TVS (mg) to mass of plastic beads samples (g).

Table 6 - TSS and TVS in different parts of the reactor.

<table>
<thead>
<tr>
<th>Part of the reactor</th>
<th>TSS [mg/g bead] ±</th>
<th>TVS [mg/g bead] ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottom</td>
<td>2.6 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Half-height</td>
<td>2.1 ± 0.3</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Top</td>
<td>1.5 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

It has been demonstrated, that concentration of biofilm strongly depends on the reactor height. At the bottom TSS was equal to 2.2 ± 0.3 mg/g, in the half-height 1.5 ± 0.2 mg/g and at the top 1.1 ± 0.2 mg/g (average values ± standard deviation of five replicates) (Table 6). These values are in the conformity with results presented in the previous studies [2,70], where amount of attached biofilm was in the range between 0.20 and 2.10 mg/g. According to [70], the limited biomass growth at the reactor top could be due to a lower value of pH and accumulation of fermentation products in this part of the reactor. After 77 days of continuous operation about 3.043 g of TVS were totally present in the reactor.

## Conclusions

The results obtained in the present study show that using anaerobic mesophilic sludge for a long term biohydrogen production in a biofilm reactor performs satisfactorily. It has been shown, that pre-treatment of the sludge at 100 °C for 1 h is an effective method to inhibit the methane-producing bacteria activity and harvest anaerobic spore-forming bacteria. In the present study biohydrogen production has been carried out in a low medium pH (inlet: 4.49 ± 0.46, outlet: 3.63 ± 0.51). This pH is the consequence of the mixing of influent and liquid recycle. Thus, the results clearly indicate the opportunity to H2 production without pH buffer addition, which is environmentally friendly and leads to significant reduce of production costs. Moreover, the present paper demonstrates the possibility to enhance HY in a dark fermentation process by using recirculation flow in APBRs. Among investigated values of HRT (from 5 to 1 h) the highest average values of HY (2.35 mol H2/mol substrate) and HPR (0.085 L h⁻¹ L⁻¹) have been obtained at HRT equal to 2 h. It has been shown, that instability of H2 production, which occurred throughout all operational periods, is supposed to be independent of the carbon source. Difficulty in obtaining stable H2 productivity could be caused by anaerobic homoacetogenic microorganisms, which are involved in the uptake of H2 and CO₂ through Wood-Ljungdahl pathway.

## Acknowledgment

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## References


